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Gene-Based Detection of Microorganisms in Environmental Samples Using PCR

John I. Glass, Elliot J. Lefkowitz, and Gail H. Cassell

Microbiology Department, The University of Alabama at Birmingham

Mark Wechser, Theresa B. Taylor, Michael Albin, and Christine Paszko-Kolva

Perkin-Elmer, Applied Biosystems Division

Monsi C. Roman

NASA Marshall Space Flight Center

ABSTRACT

Contaminating microorganisms pose a serious potential risk to the crew's well being and water system integrity aboard the International Space Station (ISS). We are developing a gene-based microbial monitor that functions by replicating specific segments of DNA as much as 10^{12} x. Thus a single molecule of DNA can be replicated to detectable levels, and the kinetics of that molecule's accumulation can be used to determine the original concentration of specific microorganisms in a sample. Referred to as the polymerase chain reaction (PCR), this enzymatic amplification of specific segments of the DNA or RNA from contaminating microbes offers the promise of rapid, sensitive, quantitative detection and identification of bacteria, fungi, viruses, and parasites. We envision a small instrument capable of assaying an ISS water sample for 48 different microbes in a 24 hour period. We will report on both the developments in the chemistry necessary for the PCR assays to detect microbial contaminants in ISS water, and on progress towards the miniaturization and automation of the instrumentation.

INTRODUCTION

Safe water to drink and air to breath are essential for human life. A critical aspect of air and water safety is the absence of pathogenic microorganisms; however the closed nature of spacecraft environments makes control of microbial contaminants all the more critical and difficult. This need is compounded by the attenuation of human immune system function due to long term exposure to microgravity[1]. To achieve control of microorganisms in spacecraft, NASA must develop environmental sensors capable of monitoring the microbial content of recycled air and water. Traditionally, analysis of environmental samples for microbial pathogens relied on culturing the organisms on suitable

growth media or propagation of viruses in tissue culture cells. Such methods are costly, slow in that some species of bacteria may take as long as 2 weeks to culture, and in many cases ineffective. Perhaps 99% of all organisms in environmental samples may not be culturable[2]. Although the current plan for monitoring microbial contamination on ISS will utilize culture methods, new technologies for microbial detection are under development that could let astronauts know in hours instead of 1-14 days if there are dangerous pathogens in their air or water. We are developing a new generation of microbial assays that rely not on the enumeration of whole bacteria or viruses, but on detection of specific biological macromolecules, such as DNA or RNA, that are unique to each organism to be detected. These assays are based on a technique called the polymerase chain reaction, or PCR.

We are in the early stages of constructing a series of quantitative PCR assays for microorganisms that a biosensor aboard the ISS should be capable of detecting (Table 1). These assays employ a fluorescent detection chemistry developed at Roche Molecular Systems and Perkin-Elmer/Applied Biosystems ABI) called the 5' nuclease assay (TaqMan™ PCR)[3], and instrumentation developed at ABI. The chemistry and instrumentation in concert are capable of determining the number of copies of a gene target that are present in a sample. Assay development takes place in a commercially available instrument, the ABI Prism® 7700 Sequence Detection System (ABI 7700)[4]. Once conditions are developed for 50 µl reactions on the ABI 7700, we will test the same assay on a prototypic micro-PCR instrument that is more characteristic of a system which could be deployed on the ISS.

PCR - DNA, or deoxyribonucleic acid, is a set of molecular instructions every organism uses to reproduce itself and its component parts. In cells, DNA is found as two long linear strands of polymerized building blocks called nucleotides. These nucleotides come in only four

Table 1. Infectious agents that are potential hazards in ISS recycled water for which a PCR based monitor should analyze.

Microorganism or Virus
1. Any Bacteria
2. Any Fungi
3. <i>Legionella sp.</i>
4. Enteric Bacteria
5. Gram Positive Bacteria
6. <i>Pseudomonas aeruginosa</i>
7. <i>Pseudomonas sp.</i>
8. <i>Mycoplasma sp.</i>
9. <i>Acinetobacter sp.</i>
10. <i>Listeria sp.</i>
11. <i>Thiobacillus sp.</i>
12. <i>Cryptosporidium</i>
13. <i>Giardia sp.</i>
14. <i>Candida albicans</i>
15. <i>Cryptococcus sp.</i>
16. <i>Histoplasma capsulatum</i>
17. Norwalk Virus
18. Hepatitis A Virus
19. Rotavirus

types, whose chemical names are abbreviated as A, T, G, and C. They are the key to both how genetic data are encoded in DNA and how the DNA molecules are faithfully replicated when cells divide. The A, T, G and C molecules make up the genetic alphabet with which the instructions needed to make a living organism are "written" in the DNA. Because of their chemical architecture, nucleotides A and T are complementary molecules that can bind only to each other. Likewise C and G are complementary nucleotides which bind only to



each other. In double-stranded DNA, as shown in the diagram above, the two strands are complements of each other because at every position along the two DNA strands, complementary nucleotides are opposite each other. When DNA is replicated, an enzyme called a DNA polymerase travels along a single DNA strand or template assembling a new complementary strand by adding the correct nucleotides one by one to the growing end of the nascent DNA molecule. Because it is a precise complement of the template strand, the newly synthesized DNA can "hybridize" to the template to form an energetically favored double-stranded DNA molecule. By utilizing the different properties of this signature molecule of life, biochemical methods have been invented which can detect segments of DNA whose nucleotide building blocks are arranged in a specific sequence.

The polymerase chain reaction, or PCR, is a technique developed by molecular biologists for

replicating a defined segment of a target DNA so that it is at an easily detectable concentration[5,6,7]. In a PCR, specific segments of DNA molecules are enzymatically replicated *in vitro* in a succession of incubation steps at different temperatures (Fig. 1). Capable of detecting a

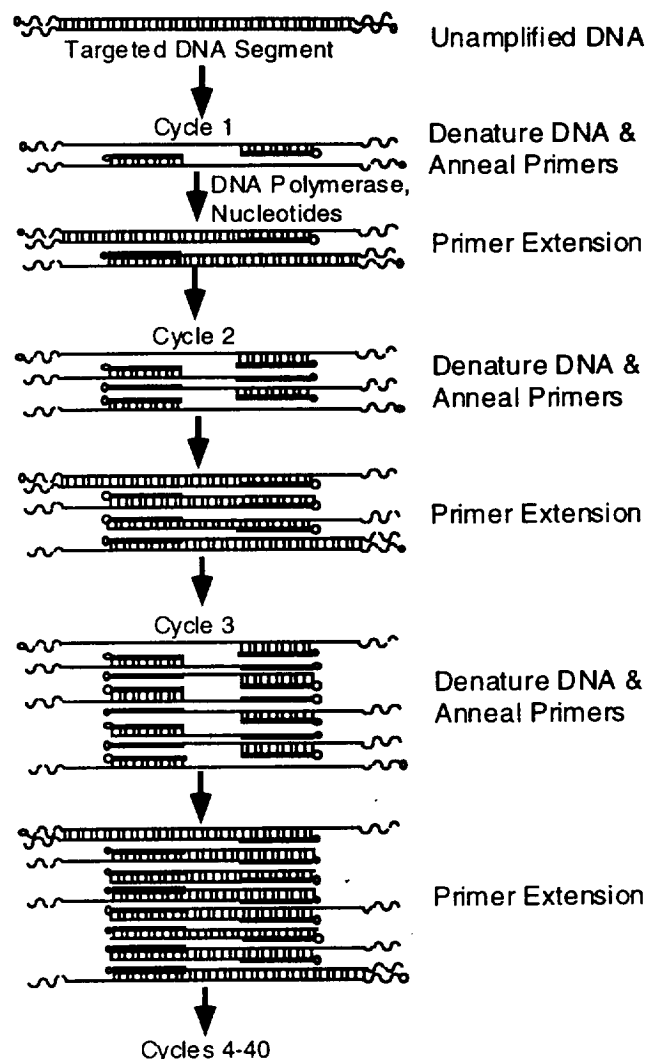


Figure 1 PCR amplification of DNA. PCRs contain template DNA, *Taq* DNA polymerase, and large molar excesses of unpolymerized nucleotides and two short pieces of single-stranded DNA called oligonucleotides or PCR primers. Typically, in a 3-step PCR, the double-stranded DNA is denatured at $\approx 95^\circ$, two oligonucleotides (the PCR primers) that are complementary to the boundaries of the target DNA segment are annealed at low temperature (typically $50-60^\circ$), and then the primers are enzymatically extended by *Taq* DNA polymerase at an intermediate temperature ($\approx 72^\circ$). A set of these 3 steps is referred to as a cycle, and the instrument that repeatedly changes the temperature of a PCR sample is called a thermocycler. The PCR process is based on repetition of this cycle and can amplify DNA segments, called amplicons, by 10^{10} to 10^{13} fold. Some PCRs use only 2 steps in each cycle by combining the annealing and extension steps.

single molecule of DNA, PCR is gaining increasing use as a microbial diagnostic method because of its unsurpassed sensitivity, specificity, and speed. As with any new scientific technique, it is continually being refined and improved.

QUANTITATIVE ANALYSIS OF PCR PRODUCTS - Culture based microbial analysis relies on the reproduction of individual organisms until sufficient progeny exist to constitute a colony that can be easily detected, and identified based on its characteristics. Similarly, PCR based microbial monitoring replicates a specific segment of a target microbe's genome to a concentration sufficient for detection and characterization. As the number of colonies on a bacterial assay plate is a quantitative function of the number of that bacteria in the sample assayed on that plate, so can the number of copies of a PCR amplified DNA sequence be a function of number of those sequences in the sample prior to PCR. It is important to note that the efficiency of amplification varies among different templates and primer sets, so quantitative PCR assays must be evaluated independently.

In most current PCR applications, to analyze post-PCR products for amplified DNA sequences, called amplicons, there are two basic methods. Most simply, the PCR products are size fractionated by gel electrophoresis, and stained with a fluorescent dye. Any amplicons present are visualized by exposing the gel to UV light. An alternative and vastly more sensitive method, often referred to as Southern blotting and hybridization, fixes any amplicons present to a substrate, usually after gel fractionation. The double-stranded DNA amplicons are then denatured and the substrate, usually a nylon membrane, is incubated with a fluorescently or radioactively labeled oligonucleotide probe. The probe specifically hybridizes to a complementary sequence of any amplicons present, and the amplicons are visualized by detecting the bound probe using either radioactivity or fluorescence detection methods. Thus probe-hybridization/PCR offers increased sensitivity and specificity over direct analysis of PCR products; however the time (hours to days) and technical requirements of both methods of post-PCR product analysis make them unsuitable for NASA's needs, and to fulfill the promise of PCR as a rapid, highly automated diagnostic tool.

For gene-based diagnostic technology to work as an effective microbial monitor the analysis of post-PCR products will have to advance beyond gel separation based methods, which are inherently slow and non-quantitative. Although the time required for the PCR thermal cycling is unchanged, a technological advance called the PCR-based 5' nuclease assay integrates detection and quantitation of PCR products with the thermal cycling. This integration of cycling and detection speeds the total PCR assay process. A diagram describing the 5' nuclease assay PCR chemistry is shown in Fig. 2. The 5' nuclease assay exploits the 5'→3' exonuclease activity of *Thermus aquaticus* DNA (*Taq*) polymerase[3, 8, 9], which digests

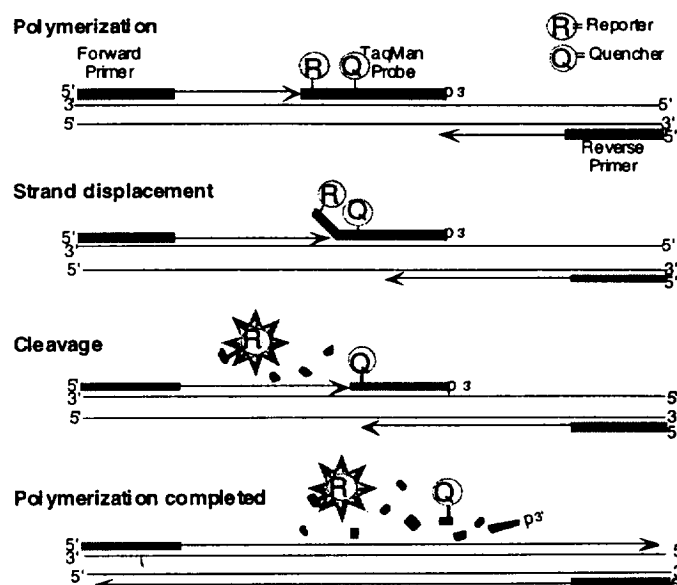


Figure 2. *Taq* DNA polymerase activity in 5' nuclease assay PCR. In a single cycle of PCR, the initial steps are template denaturation and annealing of a denatured DNA template with the forward and reverse primers, as well as with the fluorescently tagged probe (both steps not depicted). The probe is an oligonucleotide that is tagged with both a fluorescent reporter molecule (R) and fluorescent quencher molecule (Q). Subsequently, as shown here, the enzyme's polymerization dependent 5'→3' endonuclease activity frees the reporter dye from the neighbor effects of the quencher dye, so it can produce a signal that is proportional to the PCR amplification.

an internal probe labeled with a fluorescent reporter dye (FAM $\lambda_{em} = 518$ nm) and a quencher dye (TAMRA $\lambda_{em} = 582$ nm)[10]. For the intact probe, TAMRA quenches the fluorescent emission of FAM due to their spatial proximity on the probe. During PCR, the probe anneals to the target if the sequence is present, and is cleaved during extension by *Taq* polymerase. The resulting separation of reporter and quencher dyes reduces the quenching of FAM by TAMRA. The subsequent increase in FAM fluorescence is a direct consequence of target amplification, whereas the fluorescence from TAMRA, the quencher dye, remains constant irrespective of amplification. The latter point indicates that the emission from the quencher dye can be used as a normalization factor.

Because changes in FAM fluorescence are a function of amplicon accumulation, which is a function of the original PCR template concentration, the 5' nuclease assay is an inherently quantitative technique. However post run quantitative analysis of 5' nuclease assay PCRs could be inaccurate. Although amplicon accumulation during the early cycles of a PCR is exponential, as the amplicon concentration reaches 10 nM that exponential accumulation ends. Because PCR product accumulation kinetics are not constant, accurate quantitation of the number of PCR templates initially in the reaction over a

broad range of template concentrations requires the analysis be done during the PCR's exponential phase. By monitoring the progress of the 5' nuclease assay PCR after every cycle, the ABI 7700 can thus the number microorganisms in a sample can be quantitated.

ABI 7700 - This instrument is essentially a time-multiplexed laser induced fluorescence spectrograph coupled with a thermal cycler[4]. An argon laser beam is transmitted to the samples first through a dichroic mirror, then focused by a lens mounted on a rotating turret optical multiplexor, and finally onto one of 100 optical fibers. The laser energy is transmitted to the samples via 600 μm diameter fibers to a lens mounted in a specially modified cover of the thermal cycler. The 96 lenses in the cover (one over each sample tube) image the optical fibers into the samples. Both the laser generated excitation energy and the laser induced fluorescence travel through the same fibers. The fluorescence emission is imaged at the lens and travels back up the same fiber to an optical multiplexor. Images collimated by the multiplexor are reflected by the dichroic mirror onto a spectrograph (484-655 nm coverage) and CCD array. An entire tray of 96 samples can be scanned in 5 seconds as the multiplexor steps from channel to channel[4].

The fluorescence emission spectrum from each reaction is collected by the fluorimeter in real time. This total emission spectrum is the sum of the fluorescent emissions from the intact probe, the reporter (FAM) and quencher (TAMRA) dyes liberated from each other by probe cleavage, and a passive internal standard dye (ROX). The measure of probe cleavage (i.e. amplicon generation) is change in the ratio of reporter fluorescence to the fluorescence of the passive internal standard dye molecule. This value, ΔRQ , increases from cycle to cycle because reporter fluorescence increases with probe cleavage while internal standard fluorescence is invariant. At the conclusion of data collection, fluorescence(ΔRQ) is plotted against cycle number and the kinetic analysis algorithm associated with the ABI 7700 calculates the cycle at which a PCR produces a predefined level of fluorescence. That value, which is proportional to the initial template concentration, is referred to as the threshold cycle.

RESULTS & DISCUSSION

QUANTITATIVE PCR OF BACTERIA USING THE ABI 7700 - The capacity of 5' nuclease assay PCR technology to quantitatively detect bacterial DNA was tested using DNA samples extracted from the coliform bacteria *Salmonella typhimurium*. Although 5' nuclease assay PCR had been previously employed to detect *Salmonella sp.* using primers and a probe specific for the *Salmonella invA* gene, the *Salmonella* assay had not been used for quantitative PCR on the ABI 7700[11]. DNA was extracted from aliquots of *Salmonella* that had been titered by culture. To minimize the fraction of bacteria that were non-viable, the *Salmonella* culture

was in a logarithmic growth phase when the samples were collected. We employed a rigorous DNA extraction procedure in which bacteria were incubated for 20 minutes at 100° in guanidine isothiocyanate, followed by an isopropanol precipitation of the bacterial DNA. The ratio of log template concentration to threshold cycle number was linear at all template concentrations (Fig. 3). The results were essentially the same in two different experiments (each done in triplicate).

In addition to the 5' nuclease assay PCR for *Salmonella* described here have developed similar

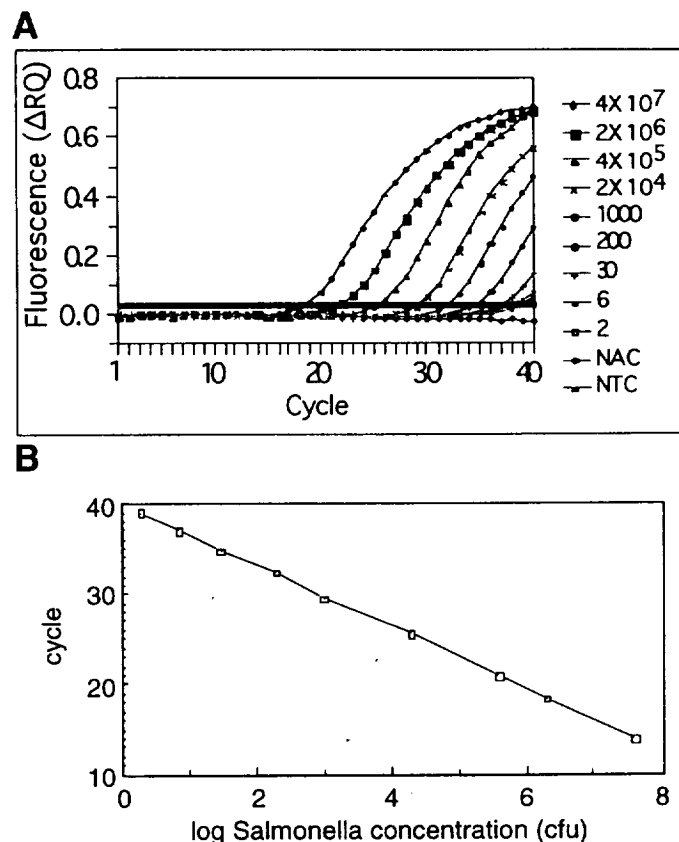


Figure 3. 5' nuclease assay PCR detection of *Salmonella typhimurium*. Aliquots of DNA isolated from varying amounts of *Salmonella* were tested in PCRs specific for the *Salmonella invA* gene[11]. The DNA was extracted using the cell lysis procedure and reagents from an Applied Biosystems EnviroAmp™ Legionella Kit. In this PCR protocol, which uses a 2-step PCR, thermocycling conditions were as follows: samples were initially held at 95°C for 10 min., then processed through 40 cycles of 95°C for 20 sec. and 61°C for 1 min., before being placed in a 4°C hold. A plot of PCR amplification (fluorescence) versus cycle number shows the progress of the reactions for the different *Salmonella* dilutions (A). The titers of the *Salmonella* used to generate the DNA samples are listed in the legend; NTC and NAC refer to "no template control" and "no amplification control" respectively. The cycle at which each PCR's fluorescence intercepts the horizontal line at $\Delta\text{RQ} = 0.03$ is the threshold cycle. The quantitative capacity of the assay is shown by the linear relationship of threshold cycle versus log *Salmonella* concentration (B).

assays for another pathogenic coliform bacteria, *Escherichia coli* O157:H7 [12,13] and for another foodborne bacterial pathogen *Listeria sp.*[14].

Universal bacterial 5' nuclease assay PCR -

One of the assays the microbial monitor must perform is to measure the total bacterial load of a sample. Because the ribosomal RNA genes in all bacterial species have diverged very little during bacterial evolution, it is possible to design PCR primers and 5' nuclease assay probes that can detect any known species of bacteria[15,16]. Although these universal bacterial PCR assays are in wide use, their capacity to detect low concentrations of bacteria is compromised by the presence of bacterial chromosomal DNA in all commercially available *Taq* DNA polymerases. *Taq* DNA polymerase is a bacterial enzyme and the contaminating bacterial DNA is leftover during enzyme purification[17].

We were uncertain whether the contaminating DNA in the *Taq* DNA polymerase we used was at a level where it would compromise the assay sensitivity needed for use on the ISS. To estimate the contaminating DNA concentration we performed PCRs using primers and a 5' nuclease assay probe designed to amplify a segment of the 16S rRNA gene from any bacteria. We did not use bacterial genomic DNA as a PCR template in this reaction. Determination of the number of living bacteria (colony forming units or cfu) in a sample is easily done; however genomic DNA from non-viable bacteria is usually an effective PCR target, and it is difficult to determine the number of non-viable microorganisms in a sample. Instead, because its concentration could be measured much more precisely, we constructed recombinant DNA PCR target for use in this experiment. This small circular molecule of recombinant DNA (≈ 5000 base pairs), called a plasmid, contained a segment of the 16S rRNA gene from the bacteria *Ureaplasma urealyticum*. PCRs were performed on a series of plasmid template dilutions (Fig. 4). In PCRs containing between 10^5 and 10^9 recombinant plasmid molecules the relationship of template concentration to threshold cycle was linear; however all template concentrations below 10^5 molecules had approximately the same threshold cycle, around 32. Thus, because of the contaminating bacterial DNA in *Taq* DNA polymerases, the threshold of detection in a universal bacterial assay (or in an assay for coliform bacteria) can be as high as 10^5 templates.

The low sensitivity of the universal bacterial PCR assay is a problem that must be resolved. There is a special preparation of *Taq* DNA polymerase that contains very low concentrations of bacterial DNA (AmpliTaq™ DNA Polymerase LD, PE Applied Biosystems); however even use of this reagent may not permit the assay sensitivity required for the ISS. Also, as described later, a variation of PCR that uses a template composed of RNA instead of DNA requires a different kind of polymerase for which low DNA versions are not available. Fortunately there are techniques to remove contaminating DNA from DNA polymerases. Methods that have been successful in other PCR applications

include eliminating the capacity of contaminating DNA to serve as a template for DNA replication by psoralen/UV radiation crosslinking[18], and nuclease digestion of the contaminating DNA by DNase I treatment followed by thermal inactivation of the nuclease[19]. Importantly, this reduction in assay sensitivity is limited to PCRs that can amplify *E. coli* DNA such as the universal bacterial assay and an assay for enteric or coliform bacteria which we have yet to develop.

5' NUCLEASE ASSAY PCRs FOR THE PARASITES GIARDIA AND CRYPTOSPORIDIUM -

We have also developed 5' nuclease assays for two waterborne parasites. *Giardia lamblia* causes giardiasis, the most common waterborne intestinal disorder found in the US[20,21]. Infections with *Giardia* are non-invasive and frequently asymptomatic. Cryptosporidiosis is a significant cause of diarrhea in man[22]. Both protozoan parasites *Cryptosporidium parvum* and *Giardia lamblia* can contaminate filtered and unfiltered drinking and recreational water supplies, and are responsible for causing gastroenteritis in humans.

Although on the ISS there will be no need for a microbial monitor to discriminate between *Giardia* and *Cryptosporidium* species that are human pathogens and those that are not, we have designed our PCR assays for these parasites so that they could have terrestrial as well as ISS uses. To achieve this primer pairs have been developed for species-specific PCR detection of *G. lamblia* and *C. parvum*. The 5' nuclease assay PCR for *G. lamblia* recognizes a sequence within the α -1-giardin gene[23]. This primer pair recognizes the target

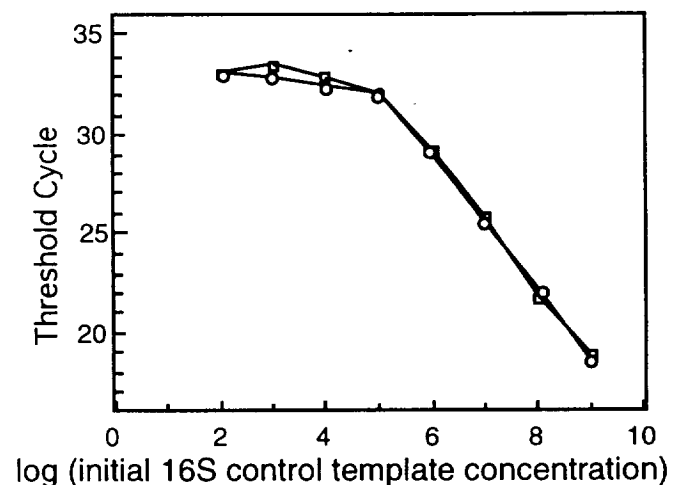


Figure 4. Universal bacterial detection assay using a 16S rRNA gene target. The relationship between initial template concentration (molecules) of 16S rRNA gene control and threshold cycle is plotted. Linearity is lost below 10^5 copies because of the presence of a similar number of contaminating DNA templates brought into the reaction with recombinant *Taq* DNA polymerase (1 μ l AmpliTaq Gold™ DNA polymerase in a 50 μ l PCR.). Data from two separate tests are plotted.

sequence in 22 different *G. lamblia* strains but does not cross react with DNA from the non-pathogenic *G. muris* and *G. ardeae*[24]. For *Cryptosporidium parvum* a 400 base pair segment of the 18S rRNA sequence was used as the target for identification[25]. This primer/probe set does not cross react with DNA from *C. muris* and *C. baileyi*, which only infect animals[22]. For both organisms, PCR detection sensitivity is ≤ 10 cysts/oocysts per reaction[25].

Because of the cell structure of these parasites makes them refractory to the sample preparation techniques that will effectively extract chromosomal DNA from most bacteria, it is necessary to employ rigorous DNA extraction protocols that use multiple cycles of freeze-thawing, proteinase K digestion, and 100° incubations in guanidine isothiocyanate.

DETECTION OF POLIO VIRUS USING 5' NUCLEASE ASSAY PCR - Among the microbes a NASA microbial monitor must be able to detect are several viruses (Table 1). Because the viruses that could pose potential problems if they were to contaminate ISS water are all significant human pathogens and difficult or dangerous to work with, we elected to begin

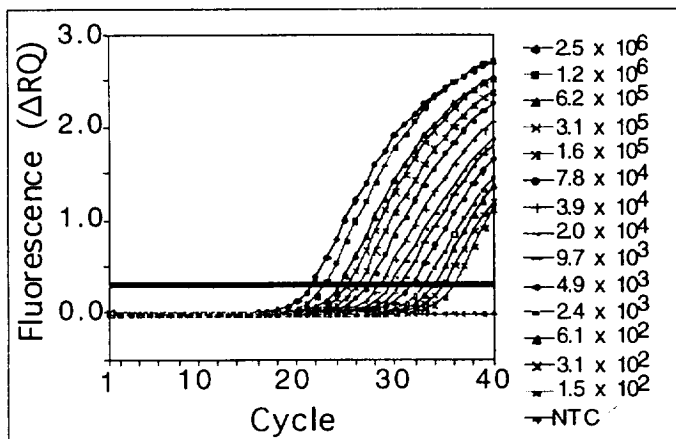


Figure 5. Detection of polio virus RNA using a 5' nuclease assay RT-PCR. Viral RNA was isolated from aliquots of polio virus using a phenol/chloroform extraction and ethanol precipitation. The precipitated viral RNA was reverse transcribed using murine leukemia virus RT at 25° for 10 minutes, 48° for 30 minutes, and 95° for 5 minutes (95° inactivates the RT). The reverse transcription product was then added to a standard 5' nuclease assay PCR using PCR primers and a 5' nuclease assay probe that amplified a 150 base segment of the polio virus 5' untranslated region. Thermocycling conditions for this PCR were 50° for 2 minutes, 95° for 10 minutes, and 40 cycles of 95° for 15 seconds and 60° for 1 minute. A plot of the threshold cycle ($\Delta RQ = 0.3$) versus the log of virus concentration was linear (data not shown). The numbers listed beside the chart are the approximate number of polio virus plaque forming units used to in each sample. NTC is a no template control.

development of 5' nuclease assay PCR assays for viruses using a less pathogenic surrogate virus, polio. Like the three viral taxons listed in Table 1, polio has an RNA genome. Because the viral genome is RNA instead of DNA as with the bacteria and parasites, it is necessary to employ a different kind of enzymatic chemistry in the polio PCR assay. PCR amplification of RNA uses a different kind of polymerase called a reverse transcriptase (RT), in addition to the *Taq* DNA polymerase. In the RT-PCR we employed, the RT is used in a pre-PCR step to make DNA copies (cDNA) of the RNA template. The cDNA is then used in a standard PCR.

There was a quantitative relationship between the number of polio virus plaque forming units used to generate the viral RNA template in a n RT-PCR and the rate of PCR amplicon generation (Fig. 5). The RT procedure we used with polio should be effective for other viruses once specific probes and primers are designed. Because we used murine leukemia virus RT it was necessary to separate the RT step to generate cDNA from the PCR step to amplify a segment of the cDNA. For RT-PCR we are also investigating the use of *rTth* DNA polymerase, which also has RT activity, because it should be possible to use *rTth* polymerase for single tube RT-PCR. The problem with *rTth* RT-PCR is the need to precisely control the concentrations of the divalent cations Mg^{++} and Mn^{++} in the reaction in order to retain both the enzyme's RT and DNA polymerase activities.

TEST OF ISS WATER FOR PCR INHIBITORS - We were uncertain whether quantitative PCR could be done on samples consisting of water reclaimed by the system designed for use aboard the ISS. In analyses performed on samples from tests of the ISS water reclamation system, which is being developed at the NASA Marshall Space Flight Center (MSFC), PCR was shown to be an effective and sensitive tool to monitor microbial contaminants[26]. However in those experiments no efforts were made to determine if there were chemicals in the reclaimed water that inhibit PCR assays and make them less sensitive[27,28]. Water from the MSFC system is highly purified however there are a number of trace chemicals present whose potential effect on the PCR enzymes is undocumented.

5' nuclease assay PCRs were done containing 5 μ l MSFC system water or 5 μ l of control water in each 50 μ l reaction. The control water was ultra-pure water prepared for PCR. Reactions were programmed with a set of serially diluted PCR templates. At all template concentrations we observed no significant difference between the reactions done with the MSFC water and the control water(Fig. 6).

MICRO-PCR - ABI has developed a prototype instrument that adapts its ABI 7700 and the 5' nuclease assay PCR technologies for use in a microvolume (2-5 μ l reaction) multiple PCR format . As with the ABI 7700 the prototype ABI micro-PCR instrument was designed for

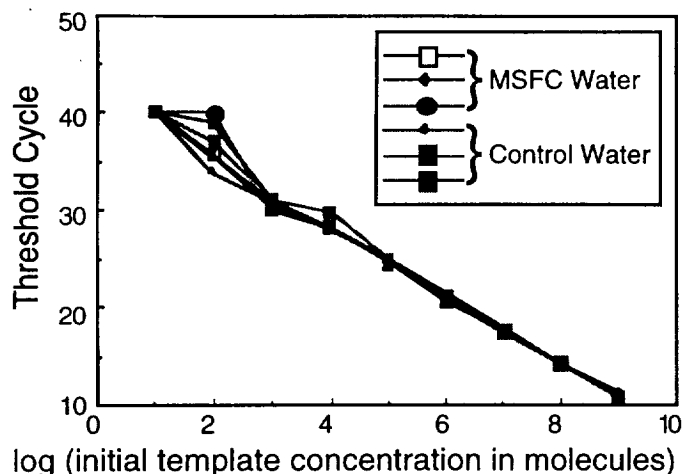


Figure 6. MSFC water does not inhibit PCR. A standard curve relating threshold cycles to initial template concentrations is shown. A *Salmonella* control DNA template diluted over 9 orders of magnitude (from 10^9 to 10 copies) was detected using the *Salmonella* PCR Amplification and Detection System when 10 percent of the reaction volume (50 μ l) contained either control water or MSFC water.

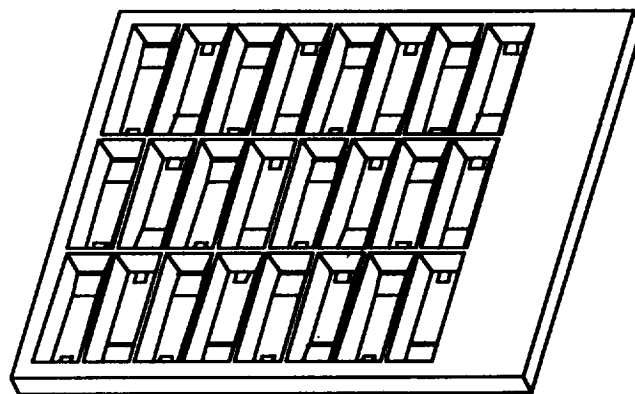
the direct quantitation of signal from a fluorescent dye labeled probe[29]. In the micro-PCR instrument a Peltier junction heating/cooling system cycles the reaction containers (microstructures) and is controlled by a microprocessor. Miniaturization of the PCR sample container is made possible by the use of modern microfabrication techniques. This has drastically reduced the amount of power required to cycle and read the sample. As the thermal cycler gets smaller it is simpler to maintain temperature uniformity and requires much less power to cycle. The instrument consists of a 75 Watt tungsten bulb for illumination and a thermo-electrically cooled CCD camera and a 5-color filter wheel for detection. Images (1:1) of the 14 mm x 14 mm microstructures are taken at several wavelengths at the end of each thermocycle. This allows the micro-PCR system to monitor the increase of the reporter fluorescence following PCR. Instead of using the 200 μ l polypropylene tubes used with the 7700. The micro-PCR instrument uses either of two kinds of microstructured reaction containers; one made of polycarbonate and the other from silicon.

The silicon glass microstructures (EG & G IC Sensors; Milpitas CA) used to run PCR were designed to be 14 x 14 mm for compatibility with the CCD imaging system employed (Fig. 7A). Each contained an array of wells equipped with a fill hole for manual addition of PCR sample and reagent, and a vent hole to allow air to escape during filling. A 0.70 mm thick silicon wafer was anisotropically etched to yield 0.5 mm deep wells. The fill and vent holes are then anisotropically etched from the opposite side of the wafer. A 4000 Å layer of thermal oxide (SiO_2) is then grown on the etched surfaces of the

wells. Finally, the chip is anodically bonded to 0.5 mm thick borosilicate glass to provide an optical window into the top of the PCR well. After the addition of sample, the chips are sealed with an acrylic-based pressure sensitive tape that is compatible with PCR chemistry (AR Care 7759, Adhesives Research; Glen Rock, PA). Volumes of these silicon microstructures, which were manually filled and tested, ranged from 0.5 μ l to 9 μ l volumes[29].

The polycarbonate plastic devices also used to run PCR were designed that have a 7 x 7 well array containing 49 wells per chip (Fig. 7B). The design includes 1mm x 1mm x 1mm wells (1 μ l volume) with a 250 micron sample distribution channel. The device includes a valve component for vacuum attachment and sample introduction. The plastic has no known material compatibility issues. Although the polycarbonate microstructure can be thermocycled more rapidly than ABI 7700 tubes, the thermal conductivity of plastic relative to silicon is a drawback for maximum cycle speed. The plastic device can be assembled without adhesives or elevated temperatures using ultrasonic

A



B

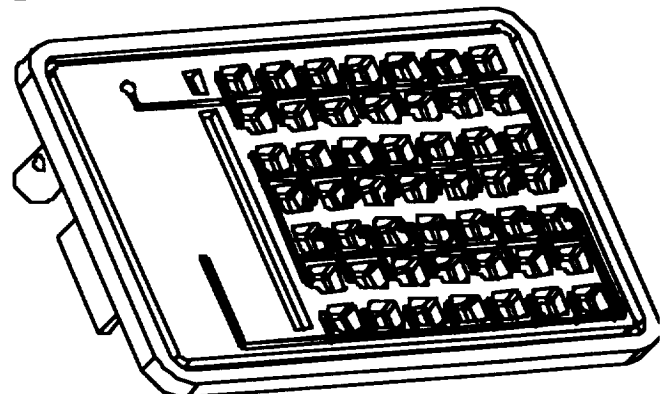


Figure 7. Silicon (A) and polycarbonate (B) PCR cassettes. The upper surface of a 24 well silicon chip configurations is shown here. The bottom of the polycarbonate cassette is very thin plastic to facilitate heat transfer. The polycarbonate cassettes would be preloaded with the PCR reagents and then desiccated for storage prior to use, the cassette contains 49 1 μ l PCR microchambers.

welding. Using an inkjet robotic delivery system, each of the 49 reaction wells is loaded with all or some of the PCR reagents. These reagents are dried down and the device assembled. The sample to be analyzed is loaded into the valve and when the vacuum seal is broken the chambers fill equally with 1 μ l of the sample.

This system of preloading and desiccating the reactants, and analyzing the PCR products in the cassette addresses two major issues that will be critical to any PCR-based monitor. First, reagent storage and delivery are accomplished; the dried reagents are stable until dissolved by the addition of the sample. The preloading eliminates the need to develop sophisticated methods of mixing several liquids in a microchamber immediately prior to thermal cycling. Containment of the PCR products inside the cassette could eliminate most of the risk of subsequent PCRs yielding false positive results because they are contaminated with old amplicons.

The capacity of the micro-PCR system to perform real time sequence specific detection of PCR products in silicon-based microstructures (Fig 7B) has been demonstrated[30]. In assays that were optimized to detect DNA sequences from the human β -globin gene and from bacteriophage lambda, equivalent PCR reaction yields, sensitivities, and specificities were observed for the silicon microstructures used with the micro-PCR system and for the larger volume polypropylene tubes used in the ABI 7700. The micro-PCR system detected a single copy of both the β -actin and lambda PCR targets[30].

There are significant differences between micro and normal devices. The ability to perform small PCR in highly conductive materials such as silicon offers the potential of rapid cycling, improved temperature uniformity, lower reagent costs, and minimal sample requirements. Analysis on the micro scale is being done in volumes as small as 0.5 μ l; whereas normal volumes for PCR using the ABI 7700 are 25-100 μ l. Miniaturization of the PCR reaction container results in significantly higher surface-to-volume ratios in the microstructures than in larger volume reaction tubes. This higher ratio may be the reason that micro-PCRs require the addition of small amounts of carrier protein (bovine serum albumin 0.05% w/v) or higher *Taq* DNA polymerase concentrations to match tube chemistry reaction performance[30]. The inclusion of carrier protein is probably needed to limit binding of the DNA polymerase to the microstructure wall surfaces. Miniaturization of PCR structures lowers the cost and the amount of wastes generated considerably because smaller amounts of reagents are being used. Importantly these microstructures can be cycled at extremely fast rates. The temperature ramp rates are increased four fold to 4°C /sec and the cycle times reduced. For the β -actin assay this results in an overall cycle time of 32.5 seconds in the chip format versus 2 minutes, 35 seconds in tubes (a five fold reduction). The 2-cycle PCR times for the microstructure were 95° - 5 seconds; 60° - 10

seconds. No significant difference in PCR yield was seen between normal and fast cycle times[30].

SUMMARY

5' Nuclease assay PCR chemistry offers the possibility of monitoring the number of microorganisms in any kind of sample. It has two major improvements over gel-based post-PCR analytic methods, and both of these advances are essential to meeting NASA's needs for a microbial monitor for the ISS.

- Although pre-PCR sample preparation and the PCR itself can require several hours to complete, the 5' nuclease assay greatly accelerates the total process of PCR-based microbial detection and quantitation relative to standard PCR diagnostic techniques. This increase in speed is possible because detection of PCR products is done in real time and integrated with the thermal cycling.
- The 5' nuclease assay PCR is an inherently quantitative technique. Within a range of template concentrations, the assay's fluorescent signal will be proportional to the amount of template present. Thus the number microorganisms in a sample can be quantitated. Any method that measures the amount of PCR amplicon generated only after the reaction is complete will be subject to errors resulting from the non-linearity of amplicon generation throughout the thermocycling.

At this early stage of our project we have both made significant progress in towards the long term goal of developing a microbial monitor for ISS and terrestrial use:

- There are no detectable inhibitors of the PCR enzymes in the recycled water generated by the MSFC water recovery system designed for deployment aboard the ISS.
- A 5' nuclease assay signal will be proportional to the amount of template present. Thus the number bacteria, parasites, or viruses in a sample can be quantitated.
- The time required for quantitative analysis of samples for microbial contaminants shortened because of the real time detection of 5' nuclease assay PCR products. Additionally the PCR process itself can be accelerated. Experiments with the prototype micro-PCR instrument using silicon-based microstructures that contained 5 μ l PCR reactions showed it could detect a single copy of bacteriophage lambda DNA in 30 minutes. Because of the high thermal conductivity of silicon, once nucleic acid had been prepared from a sample, it could be rapidly analyzed in silicon-based microstructures for a number of organisms either in parallel or in series. Although made of less thermally conductive material than silicon, the 49 well polycarbonate microdevice (Fig. 7B) could theoretically be used to analyze for that number of different kinds of microbes less than 2 hours.

Alternatively, we are considering an instrument design that would sequentially analyze for a different kind of microorganism every 30 minutes, or 48 different assays every 24 hours. This proposed device would employ flow injection analysis technology to eliminate the generation of disposable reaction vessels. PCRs would be sequentially done in a capillary tube that would replace the microdevices used in the current version of the micro-PCR instrument. The only waste product from such a detection system would be the microliter amounts of PCR product. Minimization of waste production on the ISS will be essential on long missions.

- 5' nuclease assays for the Salmonella, and the waterborne parasites, Giardia and Cryptosporidium reliably can detect as few as 10 organisms. This level of sensitivity is at the theoretical limit of detection of the technology due to the statistical uncertainty about the actual content of any sample diluted to the level of having less than 10 copies of a solute.

As we continue to develop 5' nuclease assay PCR assays for an ISS microbial monitor, we must also address these significant issues.

- An effective method must be selected to eliminate all contaminating bacterial DNA from PCR enzymes. We will test both DNase I and psoralen treatment of the enzymes to determine if either of these methods will work in our application as they have in others. This is not a major problem, and once solved our universal bacteria and coliform bacteria assays should function as well as the Salmonella and polio virus assays.
- Sample preparation is a major concern. Methods that are now routinely performed in diagnostic PCR labs may not be suitable for 5' nuclease PCR or in a fully automated microbial monitor designed for ISS use. For instance, a protocol in wide use for the preparation of clinical and environmental samples for diagnostic PCR uses a ion-chelating reagent, Chelex (Bio-Rad), to remove PCR inhibitors. This method may not be adaptable to 5' nuclease assay PCR. After processing, any residual Chelex remaining in a sample can inhibit the 5' nuclease assay reaction. Comparison of the Chelex sample preparation methods with the EnviroAmp™ method and the phenol/chloroform extraction-ethanol precipitation method suggests it may be necessary to employ an extraction procedure that uses either or both proteolytic enzymes and organic extraction of potential inhibitors (data not shown). We are beginning work with a microbial detection group at Pacific Northwest National Labs to address that issue as well as the problem of concentrating any microorganisms present in water to a level that they can be robustly detected by a PCR based monitor (≥ 100 copies of the PCR target in a 5 μ l sample).

- Our monitor will need to distinguish viable from non-viable bacteria, parasites, and fungi. PCR detection looking for DNA targets will not select for viable cells; however we are beginning a collaboration with a group from the United Kingdom who has developed 5' nuclease assay PCRs for a specific bacterial mRNAs; bacterial mRNA is an ephemeral species. Our colleagues have found it has a half life of 10 seconds in *E. coli*. We believe the current set of studies assaying for microbial DNA will give us the knowledge necessary to develop assays for viable cells by looking for specific mRNAs using the RT-PCR technology we used to amplify polio virus RNA.

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